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Reanalysis of the Usher syndrome type 2 carrier as “phenotype-free”

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Reanalysis of the Usher syndrome type 2 carrier as “phenotype-free”

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TABLE OF CONTENTS

Contents

INTRODUCTION

Auditory processing disorder (APD) is characterized by deficits in the perception of speech sounds, often leading to difficulties understanding speech in noisy environments. Individuals with APD have normal hearing thresholds and hearing ability, yet have difficulty distinguishing between similar sounds and inability to follow oral instruction. Affected individuals are easily distracted by background noise, and have trouble paying attention to verbal stimuli and comprehending complex sentences. Due to these impairments, academic performance and quality of life are often negatively impacted (BSA, 2011; Moore *et al.,* 2013). Although there is an ongoing debate regarding the definition of APD, it is believed that APD stems from neural anomalies in central auditory processing and may include difficulties in complex acoustic tasks (i.e., sound localization, pattern recognition, and temporal discrimination) (ASHA, 2005).

Over the past several years, there has accumulated a growing catalog of evidence supporting APD as a neurodevelopmental disorder. Children diagnosed as APD display symptoms that emerge throughout the course of development (Moore *et al.,* 2006), bearing a striking resemblance to other developmental disorders (i.e., dyslexia and specific language impairment (SLI)). Additionally, APD -- like several other developmental disorders -- is diagnosed only when a child fails to obtain typical levels of functioning within a specific domain, and in the absence of other known environmental causal factors (i.e., deafness, blindness, and low socio-economic status). Thus, APD diagnosis is typically made only when auditory processing deficits cannot be explained by hearing impairment or deafness (Witton *et al.,* 2010).

APD is, however, comorbid with various learning and language-related disorders, and nearly 50% of children diagnosed with APD fit a diagnosis of dyslexia, SLI, or both. (Dawes & Bishop *et al.,* 2010). For instance, Sharma *et al.,* (2009) reports that 67% of children with APD had language and reading problems, further supporting a relationship between ADP and language-related disorders. Yet, APD is not restricted to language-related disorders. Reports have shown that APD also shares significant overlap with attention-deficit disorder (ADD) (Gascon *et al.,* 1986; Cook *et al.,* 1993).

APD lacks a clear definition and diagnosis (as discussed above). This can be attributed to a failure to identify causal mechanisms, including whether the core deficits of APD arise from sub-clinical hearing impairments, atypical auditory processing and integration, comorbid cognitive disabilities (i.e., attention), or a combination. Furthermore, genetic contributions to APD are poorly understood (Witton *et al.,* 2010). Genetic studies of APD are in preliminary stages, but evidence suggests that auditory processing traits can be inherited. A recent study by Brewer *et al.,* (2016) showed significant evidence of genetic influence on non-speech auditory processing skills (i.e., temporal processing, frequency discrimination, and spectral processing), supporting the view that genetic variation can influence auditory processing. It is worth noting that Brewer *et al.,* measured the heritability of non-speech auditory processing skills, but did not access measures of speech-related auditory processing – a key component in diagnosing APD (Emanuel *et al.,* 2011). Nonetheless, studies like these can be used to create a frame work for identifying APD-risk genes.

One syndrome with promising connection to APD is Usher Syndrome (USH), a monogenic recessive disorder associated with combined bilateral hearing loss and vision loss caused by retinitis pigmentosa (RP). It is estimated that 4 in 100,000 individuals are affected by

USH (Boughman *et al.*, 1983; Keats *et al.*, 1999). Three forms of USH are observed in patients, including USH types 1 - 3 (USH1 - 3). USH1 represents a more severe version of Usher syndrome, where individuals are born deaf and RP begins pre-puberty (Otterstedde *et al.*, 2001). USH2 patients show moderate-to-severe hearing loss, with RP beginning during puberty (Reisser *et al.*, 2002). USH3 is highly variable with respect to the onset and progression of hearing and visual impairments (Pakarinen *et al.*, 1995). Extensive research has been aimed at determining and identifying causal genes associated with all three forms of Usher syndrome. To date, evidence suggests that inner ear hair cell development and retinal photoceptor stability contribute to phenotypes associated with Usher syndrome (Ahmed *et al.*, 2013; Cosgrove *et al.*, 2013; Pan *et al.*, 2012; Williams *et al.*, 2008).

Thirteen genes have been associated with Usher syndrome – six of which have been associated with USH1 (*MYO7A, USHIC, CDH23, PCDH15, USH1G,* and *CIB2)* (Weil *et al.*, 1995; Weil *et al.*, 2003; Verpy *et al.*, 2000; Bolz *et al.*, 2001; Alagramam *et al.*, 2001; Riazuddin *et al.*, 2012), three associated with USH2 (*USH2A, GPR98,* and *DFNB31*) (Eudy *et al.*, 1998; Weston *et al.*, 2004; Ebermann *et al.*, 2007), two associated with USH3 (*CLRN1* and *HARS*) (Puffenberger *et al.*, 2012), and one atypical USH gene (*PDZD7*) (Ebermann *et al.*, 2010). Although USH genes are expressed in various tissues (Bhattacharya *et al.*, 2002; Pearsall *et al.*, 2002), protein expression is strongly localized to the retina and inner ear hair cell. *USH2A,* which is responsible for roughly 55% - 90% of USH2 cases (Dreyer *et al.*, 2008; Millán *et al.*, 2011), is expressed throughout the body, including the cochlear, retina, small and large intestine, trachea, testis, and ovary, but excluding brain (Pearsall *et al.*, 2002).

Based on Usher syndrome's association with hearing loss, evidence suggests that USH2 shares a genetic etiology with APD. As mentioned above, homozygous mutations of *USH2A* are

associated with non-progressive, moderate-to-severe hearing loss and progressive RP (as seen in USH2). It is important to note that as it stands, individuals with heterozygous mutations of *USH2A* are thought to be phenotype-free and are considered unaffected carriers. However, limited research on hearing in carriers of USH2 did find that carriers had sensorineural hearing loss at low frequencies, while individuals diagnosed with USH2 displayed the expected and stereotypical high frequency hearing loss (van Aarem *et al.,* 1995). With the exception of this and a few scattered studies studies (Sondheimer *et al.,* 1979; Kloepfer *et al.,* 1966; Haas *et al.,* 1970), anomalies in USH2 carriers remain largely ignored, and the field at large continues to treat USH2 as an exclusively autosomal recessive disorder.

To further validate *USH2A* as a candidate-risk gene for APD, Perrino *et al.,* (*in-prep*) ascertained a nuclear family, all of whom were direct descendants of an individual diagnosed with APD. Affected individuals displayed characteristics similar to those of APD – i.e., problems with hearing speech sounds in the presence of background noise, a slow, distinct and deliberate style of speech, and severe language comprehension deficits, yet, all had normal hearing thresholds. Whole genome sequencing of two of the family members revealed a stop-gain mutation in the *USH2A* gene (chr1:215847632, G>A (hg19), NP_996816:p.Gln4541*) shared by all affected family members. This variation, in combination with a pathogenic variant, was previously reported as pathogenic in a unique Usher syndrome case (Le Quesne Stabej *et al.,* 2012). Together, these results suggest a relationship between *USH2A*, APD and language development.

Usherin, the protein product of *USH2A*, is found in the basement membrane of differentiating stereocilia acting as a lateral ankle link connecting adjacent stereocilia (Adato *et al.,* 2005). Research conducted by Liu *et al.,* (2007) furthered our understanding of usherin, with

data showing that usherin is essential for postnatal maturation of cochlear hair cells. During the development of the cochlea, kinocilium provide important positional cues for subsequent stereocilia development. Once the cochlea reaches maturation, kinocilium recede, leaving stereocilia in their correct, final location. Lui *et al.*, suggested that usherin is involved in this interaction between stereocilia and kinocilium, as well as the interaction between adjacent stereocilia. Results from a mouse model of Usher syndrome, involving a knockout of the rodent homolog of *USH2A* (*Ush2a*) revealed that loss of usherin results in the disorganization of outer cochlea hair cells. This could explain the hearing loss associated with USH2 (Liu *et al.*, 2007), as well as a potential relationship with APD (i.e., central auditory processing abnormalities).

The current study was designed to further explore the relationship between *USH2A*, ADP and language development, with an emphasis on auditory processing ability. Previous studies of *Ush2a* KO mice have already reported morphological abnormalities within the cochlea. Specifically, the localization of usherin in all outer hair cell layers of the cochlea was absent in KO mice, resulting in a lack of outer hair cells in the basal cochlea. Additionally, mean cochlear thresholds by measure of DPOAEs indicated hearing loss for high-frequency sounds (>20,000 Hz) in *Ush2a* KO mice. This phenotype was observed at both 4 and 7 months, suggesting the observed high-frequency hearing loss was non-progressive -- a phenotype consistent with human USH2 (Liu *et al.*, 2007). Although these studies provided excellent measures of morphological differences between wildtype (WT) and *Ush2a* KO mice, they failed to examine how a heterozygous mutation of *USH2A* might impact hearing and auditory processing ability. Our goal was to further explore the relationship between *USH2A*, ADP and language development, by examining both *Ush2a* heterozygous (HT) and KO mice on a battery of rapid auditory processing tasks, as well as completing a characterization of their ultrasonic vocalizations.

Strong evidence suggests that rapid auditory processing (RAP) ability is associated with language development, and deficits in RAP ability are robust predictors of deficits in language ability in humans (Choudhury *et al.,* 2007; Benasich & Tallal, 2002; Benasich *et al.,* 2006). The results presented here suggest that studies of USH-related genes can provide a novel approach to understanding the genetics associated with auditory processing disorder, with implications for screening both APD and language outcomes.

METHODS

Subjects

Six *Ush2a* knockout (KO) male mice were provided by Dr. Jun Yang (University of Utah; Liu et al., 2007), and were re-derived on an 129S4/SvJaeJ background strain at the Gene Targeting and Transgenic Facility (GTTF) at UConn Health. F1 subjects were delivered to the University of Connecticut where they were crossed with six wildtype (WT) controls (129S4/SvJaeJ; stock number 009104) purchased from The Jackson Laboratory (Bar Harbor, ME). The resulting F2 offspring were heterozygous (HT) for the *Ush2a* gene. Breeding pairs (HT x HT) were used to generate the experimental subjects, such that all genotypes (homozygous knock-out, heterozygous, and wildtype) were represented within-litter (F3). F3 genotypes were determined via PCR of ear punch DNA using the following primers: Common (5' – GTGAATACAGGCACCTCTGAATGTGAC – 3'), WT (5' – GTCACGGCTGAATCCCGAAGC – 3'), KO (5' – GAGATCAGCAGCCTCTGTTCCAC – 3'). Twelve WT male mice, 12 HT male mice, and 11 *Ush2a* KO male mice from F3 were randomly selected in adulthood for behavioral testing. All subjects were single housed in standard Plexiglass mouse-tubs (12hr / 12hr light-dark cycle), with food and water available *ad libitum*. Behavioral testing was performed blind to subject genotype. All procedures were conducted in

compliance with the Guide to the Care and Use of Laboratory Animals and was approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

Auditory Processing (Postnatal day (P)65 – P112)

Modified Prepulse Inhibition Paradigm

Following puberty, subjects were tested on a battery of auditory processing tasks using a modified prepulse inhibition paradigm (see Fitch et al., 2008 for review). The ability to suppress an acoustic startle response (ASR; an involuntary, reflexive response to an unexpected auditory stimulus [startle eliciting stimulus (SES); 105dB, 50ms, broadband white noise burst (1 kHz – 10 kHz)]) was measured. Subjects were placed on cell-loaded platforms (Med Associates, St. Albans, VT), and presented with varying auditory stimuli generated via RPvdsEx software and a RZ6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Subject motor reflex responses were recorded via a Biopac MP150 acquisition system and Acqknowledge 4.1 software (Biopac Systems, Goleta, CA) connected to the load cell platforms. During cued trials, subjects were presented with an auditory cue (prepulse) 50ms before the presentation of the SES (no cue presentation occurred during uncued trials). If the subject was able to detect the auditory cue, an attenuation (or reduction) of their ASR was expected relative to their ASR during an uncued trial. If the auditory cue was not detected, the response was expected to equate to an uncued trial. Quantification of this phenomenon was termed the "attenuation score" (ATT), which compared the mean amplitude of cued ASR to that of the uncued ASR for each subject, for each session condition.

$$\frac{mean\ cued\ ASR}{mean\ \ uncued\ ASR} x\ 100$$

Normal Single Tone

Subjects were first tested on Normal Single Tone (NST) to measure baseline prepulse inhibition, general auditory ability, and to rule out any underlying auditory processing impairments that might impede performance on subsequent auditory processing tasks (i.e., impaired reflex mechanics). Subjects were required to detect a simple single tone (50ms, 75dB) against a silent background. This cue was presented 50 ms before the SES on half of the trials (104 cued and uncued trials in total, pseudorandom and evenly distributed), at inter-trial intervals (ITI) ranging from 16s – 24s. Two versions of this task were developed – a 15kHz version (cue; 50ms, 75dB, 15,000 Hz tone) and a 40 kHz version (cue; 50ms, 75dB, 40,000 Hz tone). All subjects were able to perform both versions of the task (15 kHz – P65; 40 kHz – P103). The frequency-matched NST score for each subject was used as a covariate in the analysis of further tasks, specifically to eliminate individual differences in PPI or hearing from subsequent auditory processing analyses.

Embedded Tone

The variable duration Embedded Tone Task (EBT) consisted of 300 pseudorandom trials with ITIs ranging from 16s – 24s. Subject's ability to detect a change in tone frequency from a constant pure-tone background was measured, and ATT scored were calculated. During cued trials, a single cue was presented 100ms before the SES; for uncued trials, the "cue" was presented 0ms before the SES (i.e., no cue). Three versions of this task were used: 1) a long-duration EBT task, where the cue duration ranged from 0ms – 100ms (cue; 75dB, 5600 Hz tone & pure-tone background; 75dB, 10,500 Hz tone); 2) a short-duration EBT task, where the cue duration ranged from 0ms – 10ms (cue; 75dB, 5600 Hz tone & pure-tone background; 75dB, 10,500 Hz tone); 3) an ultrasonic long-duration EBT task where the cue duration ranged from

0ms – 100ms (cue; 75dB, 35,000 Hz tone & pure-tone background; 75dB, 40,000 Hz tone). This combination of frequencies and temporal durations was designed to capture the range of processing capacities, allowing us to test for genotype-specific differences in that range. Non-ultrasonic and ultrasonic versions of the task were necessary to determine any Genotype effects observed were frequency dependent. Both non-ultrasonic versions of the task were administered for five consecutive days, and the ultrasonic version of the task was administered for four consecutive days (EBT 100: P68 – P72; EBT 10: P75 – P79; EBT 100 at 40kHz: P104 – P107).

Silent Gap

The Silent Gap (SG) task was used to evaluate the ability to detect silent breaks (or silent gaps) in a continuous white noise background (75dB, 100 Hz – 10,000 Hz). Each testing session consisted of 300 pseudorandom cued or uncued trials, with an ITI ranging from 16s – 24s. During cued trials, a silent gap was presented 100ms before the SES. The cue durations ranged from 0ms – 300ms (SG 0-300) or 0ms – 100ms (SG 0-100). For uncued trials, the silent gap consisted of a 0ms cue. Each version of the task (SG 0-300 and SG 0-100) was conducted for five consecutive days (SG 300: P82 – P86; SG 100: P89 – P93).

Pitch Discrimination

Pitch Discrimination (PD) testing assessed the subject's ability to detect subtle changes in pitch within a constant pure-tone background. Each testing session consisted of 300 pseudorandom trials, with an ITI ranging from 16s – 24s. During cued trials, the cue was presented for 300ms, 100ms before the SES. "Cues" presented during uncued trials were presented at the same frequency as the pure-tone background. Two versions of this task were used for this study: 1) PD task where the cue frequency deviated 5 Hz – 75 Hz above or below a 10,500 Hz pure-tone background (cue: 300ms, 75dB tone & pure-tone background: 10,500Hz

tone); and 2) ultrasonic PD task where the cue frequency deviated 5 Hz – 75 Hz above or below a 40,500 Hz pure-tone background (cue: 300ms, 75dB tone & pure-tone background: 40,500Hz tone). A non-ultrasonic PD task was administered for five consecutive days, and an ultrasonic PD task was administered for three consecutive days (PD: P96 – P100; PD at 40kHz: P110 – P112).

Ultrasonic Vocalizations (USVs; P117 – P121)

Following assessment of auditory processing ability, ultrasonic vocalizations (USVs) were recorded and analyzed using methods adapted from Chabout *et al.*, 2017. Using WT female homecage bedding and urine collected 5 days prior to testing, a single experimental male mouse was placed in a standard Plexiglass tub with a single novel WT female mouse and allowed to freely interact for 5 minutes. In this setting, a male mouse will vocalize while the female does not, such that recoded calls can be attributed to the male. A Brüel & Kjær Type 4954-B microphone (Brüel & Kjær, Nærum, Denmark), connected to a RME Fireface UC audio interface (RME Audio, Haimhausen, Germany), was placed 5cm above the top of the Plexiglass tub. USVs were recorded at 192,000 Hz using DIGICheck 5.92 (RME Audio, Haimhausen, Germany) to ensure all USVs were captured. Following USV recording, sound files (*.wav*) were analyzed in MATLAB (MathWorks) using MUPET (Mouse Ultrasonic Profile ExTraction (Van Segbroeck *et al.*, 2017). Syllables in the range of 35,000 Hz to 110,000 Hz, and durations between 8 ms to 200 ms, were analyzed. If syllables occurred less than 5 ms apart, they were excluded from analyses. Following these parameters, a syllable repertoire was generated, illustrating 40 unique syllables (Figure 1). These 40 unique syllables were then assigned to one of ten potential syllable categories, as defined by Heckman *et al.*, 2016. From our subjects, only eight of the ten possible categories were produced; Short, Down-FM, Up-FM, Chevron, Flat, 1-

Freq Step, Noisy, and Complex -- 2-Frequency Step and Reverse Chevron were not observed (Heckman *et al.,* 2016). Total time spent vocalizing (s), Duration of syllables (ms), and the mean frequency (kHz) of each syllable was exported from MUPET and used for statistical analyses.

Auditory Processing – Statistical Analysis

Normal Single Tone attenuation scores were analyzed using a one-way analysis of variance (ANOVA) comparing WT, HT, and *Ush2a* KO performance. To account for individual variation in baseline prepulse inhibition and hearing, NST was used a covariate for subsequent statistical analyses (NST 15 kHz was used as a covariate for all non-ultrasonic auditory tasks; NST 40 kHz was used as a covariate for all ultrasonic auditory tasks). EBT, SG, and PD tasks were analyzed using a mixed factorial design. Differences in ATT scores for non-ultrasonic EBT 100 and EBT 10 were conducted using a 3 x 5 x 9 repeated measures ANCOVA, with Genotype (three levels; WT, HT, *Ush2a* KO) as the between-subjects variable, and Day (five levels) and cue Duration (nine levels) as the within-subjects variables. Ultrasonic EBT 100 data was analyzed using a 3 x 4 x 5 repeated measures ANCOVA with Genotype (three levels) as the between-subjects variable, and Day (four levels) and cue Duration (five levels) as the within-subjects variables. Similar to non-ultrasonic EBT, SG 300 and SG 100 were analyzed using 3 x 5 x 9 repeated measures ANCOVA, with Genotype (three levels; WT, HT, *Ush2a* KO) as the between-subjects variable, and Day (five levels) and cue Duration (nine levels) as the within-subjects variables. For Pitch Discrimination, a 3 x 5 x 9 and a 3 x 3 x 5 (for non-ultrasonic PD and ultrasonic PD, respectively) repeated measures ANCOVA was used to determine ATT differences, where Genotype (three levels) was the between-subject variables and Day (five

levels, three levels) and Frequency (nine levels, five levels) were the within-subject effects. Statistical analyses were completed used SPSS 24 with an alpha criterion of 0.05.

Ultrasonic Vocalizations – Statistical Analysis

The time spent vocalizing, number of calls produced, duration of syllables produced, volume of calls, and mean frequency of calls (both overall and collapsed across syllable category) was analyzed using a one-way analysis of variance (ANOVA) comparing WT, HT, and *Ush2a* KO scores.

RESULTS

Auditory Processing

Ush2a HT, KO, and WT control mice were tested on a series of acoustic detection tasks adapted to a prepulse inhibition (PPI) paradigm, specifically to index hearing and complex acoustic discrimination (Fitch *et al.,* 2008). Subjects were first assessed for detection of pure-frequency tones of variable frequency (15 or 40 kHz) via PPI reflex. Results at 15kHz showed that *Ush2a* HT mice were significantly worse than both WT controls and *Ush2a* KOs [(Overall): $F(2,32) = 3.697$, $p < 0.05$; (WT vs. HT): $F(1, 22) = 3.201$, $p < 0.10$], while WT and *Ush2a* KO did not differ [(WT vs. KO): $F(1, 21) = 1.054$, $p > 0.05$; (HT vs. KO): $F(1, 21) = 5.016$, $p < 0.05$] (Figure 2). At 40kHz, *Ush2a* HT mice performed similarly to WT control, whereas *Ush2a* KO mice trended to the expected impairment [(Overall): $F(2,32) = 1.995$, $p < 0.1$, one-tail; (WT vs. HT): $F(1, 22) = 0.619$, $p > 0.05$; (WT vs. KO): $F(1, 21) = 3.125$, $p < 0.10$; (HT vs. KO): $F(1, 21) = 1.517$, $p > 0.05$] (Figure 2). In order to determine whether higher-order acoustic deficits reflected simple hearing loss, individual scores on this single tone task (frequency-matched) were used as a covariate for analysis of complex tasks. Findings showed that even when low-level hearing loss was factored out, using NST as a covariate, deficits were still evident for HT

mice on complex low frequency tasks [Embedded Tone 100: 10.5 kHz (Overall): $F(2, 31) = 3.691$), $p < 0.05$; (WT vs. HT): $F(1, 21) = 3.634$, $p < 0.10$; (WT vs. KO): $F(1, 20) = 0.691$, $p > 0.05$; (HT vs. KO): $F(1, 20) = 6.845$, $p < 0.05$] (Figure 3), [Embedded Tone 10: 10.5 kHz (Overall): $F(2, 31) = 4.635$, $p < 0.05$), (WT vs. HT): $F(1, 21) = 2.798$, p =0.10; (WT vs. KO); $F(1, 20) = 1.769$, $p > 0.05$; (HT vs. KO): $F(1, 20) = 13.320$, $p < 0.05$] (Figure 4), [Embedded Tone 100: 40 kHz (Overall): $F(2, 31) = 0.948$, $p > 0.05$] (Figure 5). KO mice displayed deficits on higher frequency tasks [Pitch Discrimination: $F(2, 31) = 1.325$, $p > 0.05$] (Figure 6),[Pitch Discrimination: 40.5 kHz (Overall): $F(2, 31) = 2.335$, $p > 0.05$; (WT vs. HT): $F(1, 21) = 0.247$, $p > 0.05$; (WT vs. KO): $F(1, 20) = 9.232$, $p < 0.05$; (HT vs. KO): $F(1, 20) = 1.657$, $p > 0.05$] (Figure 7). There were no genotype differences on SG tasks [Silent Gap 300: $F(2, 31) = 2.497$, $p > 0.05$] (Figure 8), [Silent Gap 100: $F(2, 31) = 1.378$, $p > 0.05$] (Figure 9).

Ultrasonic Vocalizations

Ultrasonic vocalizations (USVs) were recorded from *Ush2a* HT, KO, and WT mice and analyzed to determine Genotype-based differences in USV properties, including time spent vocalizing, number of calls, duration of syllables, volume of produced syllables and pitch. There were no significant Genotype differences on total time spent vocalizing [$F(2, 237) = 0.6304$, $p > 0.05$] (Figure 10) and total number of calls produced [$F(2, 269) = 1.3808$, $p > 0.05$] (Figure 11). No call-specific analyses showed overall or pair-Genotype effects for time spent vocalizing and number of calls. However, *Ush2a* HT mice produced significantly shorter calls than WT mice [$F(2, 16100) = 26.70$, $p < 0.05$] (Figure 12). Additionally, *Ush2a* HT mice produced USVs with significantly more energy than WT mice (a measure of syllable volume) [$F(2, 16100) = 142.54$, $p < 0.05$] (Figure 13). Finally, results showed that *Ush2a* HT mice vocalized at significantly higher frequencies (pitch) [$F(2, 16100) = 87.476$, $p < 0.05$] (Figure 14. (*See Tables 1, 2, and 3*

for a compressive statistical summary of duration of syllables, volume of produced syllables, and frequency of produced syllables. The syllable repertoire generated by MUPET can be seen in Figure 1).

Together, these findings indicate that heterozygous disruption of the *Ush2a* gene associates with low-frequency hearing loss, a phenotype distinct from the high-frequency hearing loss characterizing Usher syndrome in humans, as well as the phenotype observed in KO mice. The low-frequency hearing loss in HTs was further associated with higher-order auditory processing deficits, even after co-varying for the hearing loss. Moreover, this low-frequency hearing loss was associated with modifying effects (possibly developmental) on adult expressive vocalizations.

DISCUSSION

We assessed rapid auditory processing (RAP) ability and conducted a complete characterization of ultrasonic vocalizations (USV) in a mouse model of Usher syndrome type 2 in efforts to better understand the relationship between the *USH2A* gene, and auditory processing disorder (APD), with implications (in humans) for language development. Results showed that *Ush2a* KO mice displayed the expected high frequency hearing loss, as seen on NST 40 kHz and PD 40 kHz tasks, while *Ush2a* HT mice displayed low-frequency hearing loss on NST 15 kHz and frequency-driven deficits on low-frequency RAP tasks (i.e., EBT 100 at 10.5 kHz and EBT 10 at 10.5 kHz). *Ush2a* HT and KO mice did not show deficits or impairments on temporally-driven RAP tasks (i.e., SG 300 and SG 100), suggesting that the auditory processing impairments are restricted to the frequency domain. Additionally, *Ush2a* HT mice produced USVs that were shorter in duration and at a higher frequency than WT mice. Not only do these findings support the role of *USH2A* in high-frequency hearing loss, consistent with the diagnostic criteria for

USH2, but they introduce a novel phenotype for USH2 carriers – heterozygous mutations of *USH2A* result in low-frequency, low-level hearing loss, which in-turn leads to higher-order auditory processing deficits, coupled with communicative deficits.

Ush2a HT mice produce shorter ultrasonic vocalizations (USV) at higher-frequencies than WT mice. Recent research in studying and understanding mouse USVs revealed that these calls reflect the emotional and behavioral state of the mouse (Chabout *et al.*, 2012), as male mice have been shown to produce context-specific USVs. The lack of significant differences between WT, HT and KO *Ush2a* mice on measures of time spent vocalizing and number of calls could suggest that these mice do not display social deficits. However, because differences in the duration of call types, frequency of calls, and the volume of the produced calls were seen in *Ush2a* HT mice, we have reason to believe that expressive communication ability was impaired. These results parallel human clinical findings in which USH carriers (heterozygous for the mutation) have a distinct style of speech (Perrino, *in-prep*). The alterations in vocalizations may be a consequence of the auditory processing deficit associated with this genetic variation, since auditory feedback is crucial for vocal communication.

To further understand the role of heterozygous mutations in *USH2A*, more comprehensive phenotyping is required. Liu *et al.*, (2007) characterized the morphological differences between WT and *Ush2a* KO mice. Unfortunately (and for reasons understood), HT mice were not examined. Results from that study show a lack of usherin in the basal cochlea in *Ush2a* KO mice, resulting in a disorganized and malformed hair cell bundles. This area of the cochlea is responsible for hearing high-frequency sounds and, if damaged or underdeveloped, would explain the high-frequency hearing losing seen in USH2 patients. In both the WT and *Ush2a* KO, the middle cochlear was intact. An important follow-up study would be to examine

the cochlear morphology of *Ush2a* HT mice. Our evidence implies that *Ush2a* HT mice would have disorganized middle or apical basilar membrane and hair cell morphology, which would explain the low-frequency hearing deficits. Further testing would be needed to support these claims.

The results presented here are some of the first to show auditory processing impairments in *Ush2a* HT mice. However, future studies are required to more accurately detail the frequency-specific hearing loss in these *Ush2a* carriers. Our Normal Single Tone task utilized two frequencies, 15 kHz and 40 kHz. It would be naïve to assume that these are the only two frequencies that evoke the observed behavioral deficits, and more comprehensive phenotyping is warranted to determine upper and lower boundaries of the frequency-based loss. In addition to comparing the structural differences between WT and *Ush2a* KO cochlear morphology, Liu *et al.,* (2007) measured mean cochlear thresholds and found *Ush2a* KO mice showed high-frequency hearing loss (Liu *et al.,*2007). These same distortion product otoacoustic emissions (DPOAE) measures could be used on our *Ush2a* HT mice to determine the specific frequency range of hearing difficulties. Together, modifications to previously used methods (i.e., altering the frequency of NST tasks) and the addition of several other auditory related tasks would provide additional insight into the behavior associated with *Ush2a* HT mice.

Our results have important clinical implications, especially for individuals who are carriers of USH2 mutations. Efforts have been made to develop physiological tests and/or markers for carrier detection but have all failed to accurately identify individuals. These measures include dark adaption rod/cone thresholds, ophthalmoscopic abnormalities, and speech discrimination scores (van Aarem *et al.,* 1995; Sondheimer *et al.,* 1979; de Haas *et al.,* 1970). Based on the results presented above, a better method for accurate carrier detection may be to

exploit the auditory-evoked startle response. Infants as young as 2 months old have the ability to perceive and identify rapidly presented acoustic stimuli, an ability necessary for language development (Aslin *et al.,* 1989; Jensen *et al.,* 1993; Jusczyk *et al.,* 1980). Suspected carriers of USH2 could be tested for rapid auditory processing impairments using methods illustrated in Benasich & Tallal (2002). This human task mirrors the higher-order auditory processing tasks that were used to assess *Ush2a* HT mouse auditory processing ability and would also (in humans) provide predictions on later-language outcomes, as rapid auditory processing ability is known to predict language development (Benasich & Tallal, 2002).

Overall, these findings support the view that *USH2A* plays a role in APD and possibly language development. Additional evidence from large human genetic samples suggest that hearing loss associated with variants in *USH2A* may act as a modifying risk factor for APD and language disorders when other language-related mutations are present (UK ALSPAC sample, Perrino *et al., in-prep*). Genes implicated appear to cluster to genetic mediation of synaptogenic and neuronal migration pathways, suggesting a possibility for interaction between low-level hearing loss associated with variants on a gene not expressed in brain (*USH2A*), and higher-order neurodevelopmental gene mutations that do affect the brain, together affecting language outcomes. These mouse results, however, suggest that even low-level hearing anomalies associated with altered gene expression in the cochlea, but not the brain, may be sufficient to developmentally impact more complex and higher-order auditory processing, and even expressive vocalizations. Future research will continue efforts to tease apart genetic contributions to hearing, APD, and developmental anomalies of language.

CONCLUSION

Here we not only confirm that homozygous mutations of *USH2A* result in high-frequency hearing loss (similar to Usher syndrome type 2) but provide novel insight into *USH2A* heterozygous mutations and associated low-frequency hearing loss and altered communication ability. Results urge updates to how we approach screening and treatment of individuals with *USH2A* variants, and USH carriers.

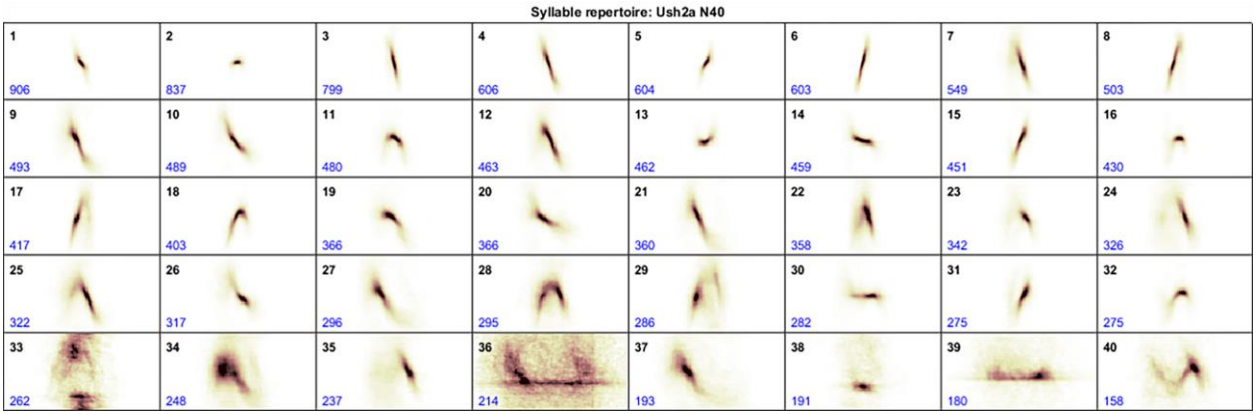

Figure 1 – Syllable Repertoire generated by MUPET
Forty unique syllables generated via MUPET analysis. Each syallable was assigned to one of ten possible categories (Heckman *et al.*, 2016). Only eight of the ten categories were observed in the current repertoire. The number in the bottom left corner indicates the count of utterances seen across all subjects.

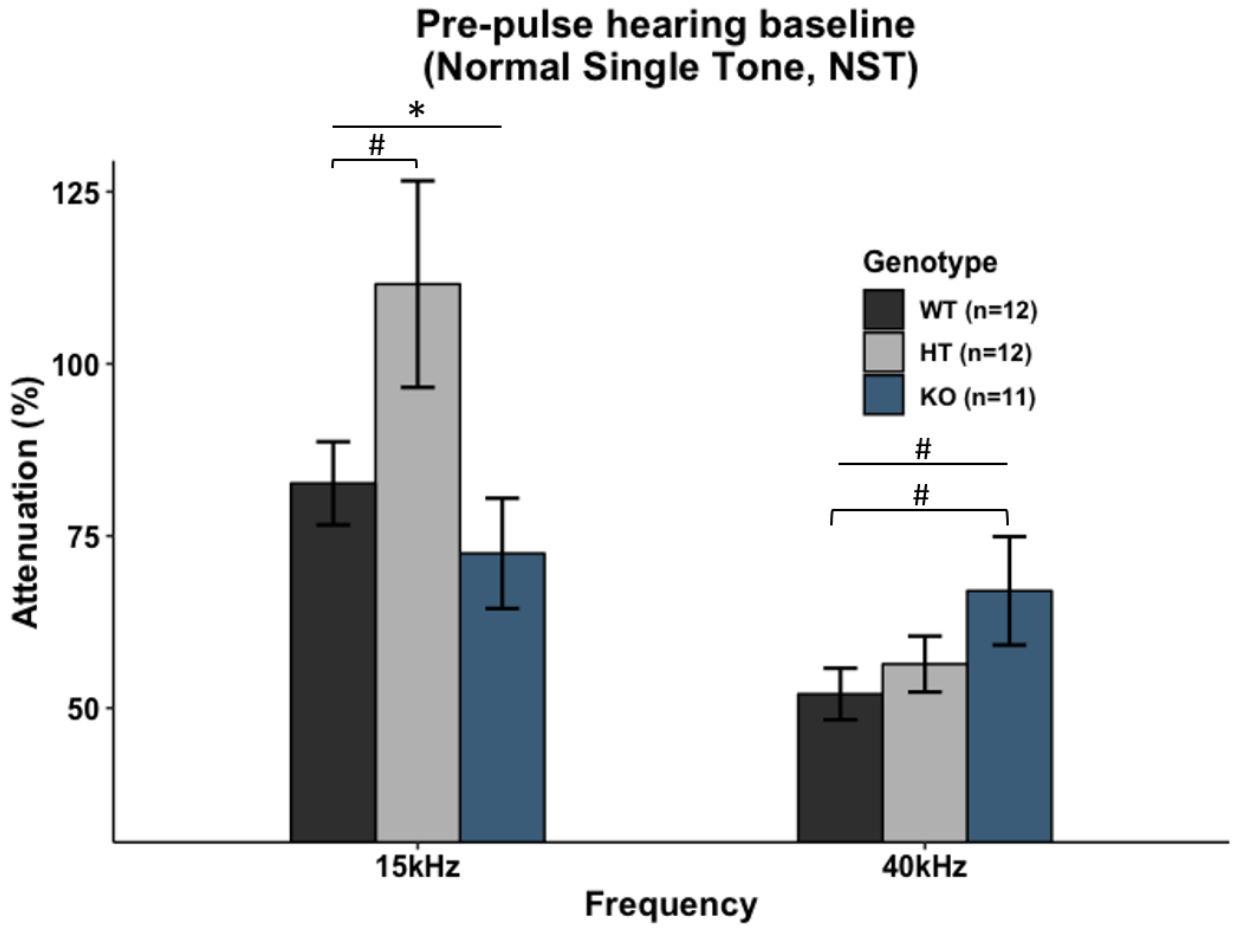

Figure 2 – Normal Single Tone (NST) attenuation scores
A main effect of Genotype was found for NST 15kHz [(Overall: 15kHz): $F(2,32) = 3.697$, $p < 0.05$], with *Ush2a* HT mice trending towards worse performance than WTs. There was a marginally significant effect of Genotype on NST 40kHz [(Overall: 40kHz): $F(2,32) = 1.995$, $p \leq 0.1$], with *Ush2a* KO mice displaying poor performance than WTs. (#$p \leq 0.10$, *$p < 0.05$). *Lower attenuation scores indicate better performance*.

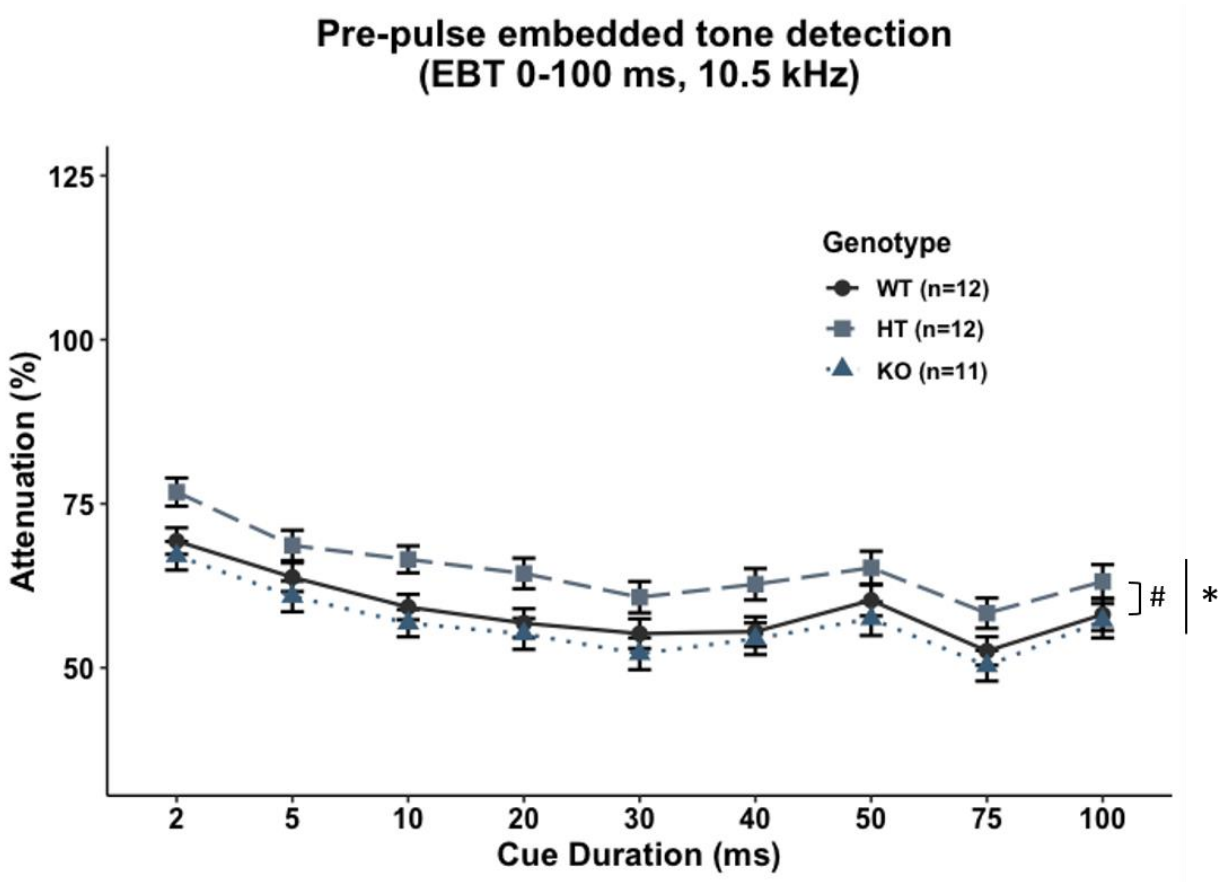

Figure 3 – Embedded Tone (EBT) 0-100 at 10.5kHz averaged across days
A main effect of Genotype was found [(Overall): $F(2, 31) = 3.691$), $p < 0.05$]. *Ush2a* HT mice showed poorer performance on EBT 0-100 at 10.5 kHz compared to WT controls ($p \leq 0.10$), using NST 15kHz as a covariate. (#$p \leq 0.10$, *$p < 0.05$). *Lower attenuation scores indicate better performance*.

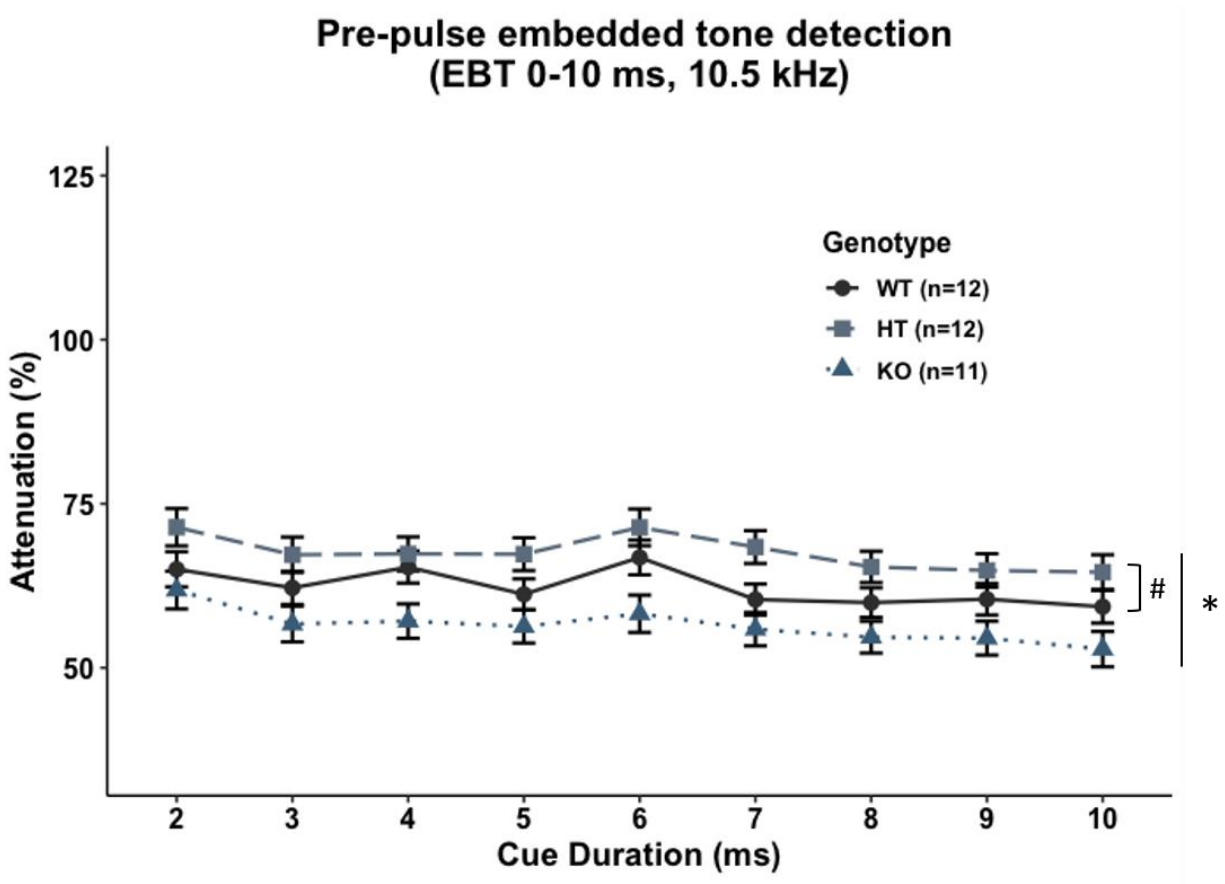

Figure 4 – Embedded Tone (EBT) 0-10 at 10.5kHz averaged across days
A main effect of Genotype was found [(Overall): $F(2, 31) = 4.635$, $p < 0.05$]. *Ush2a* HT mice displayed poorer performance on EBT 0-10 at 10.5 kHz compared to WT controls ($p \leq 0.10$), using NST 15kHz as a covariate. (#$p \leq 0.10$, *$p < 0.05$). *Lower attenuation scores indicate better performance*.

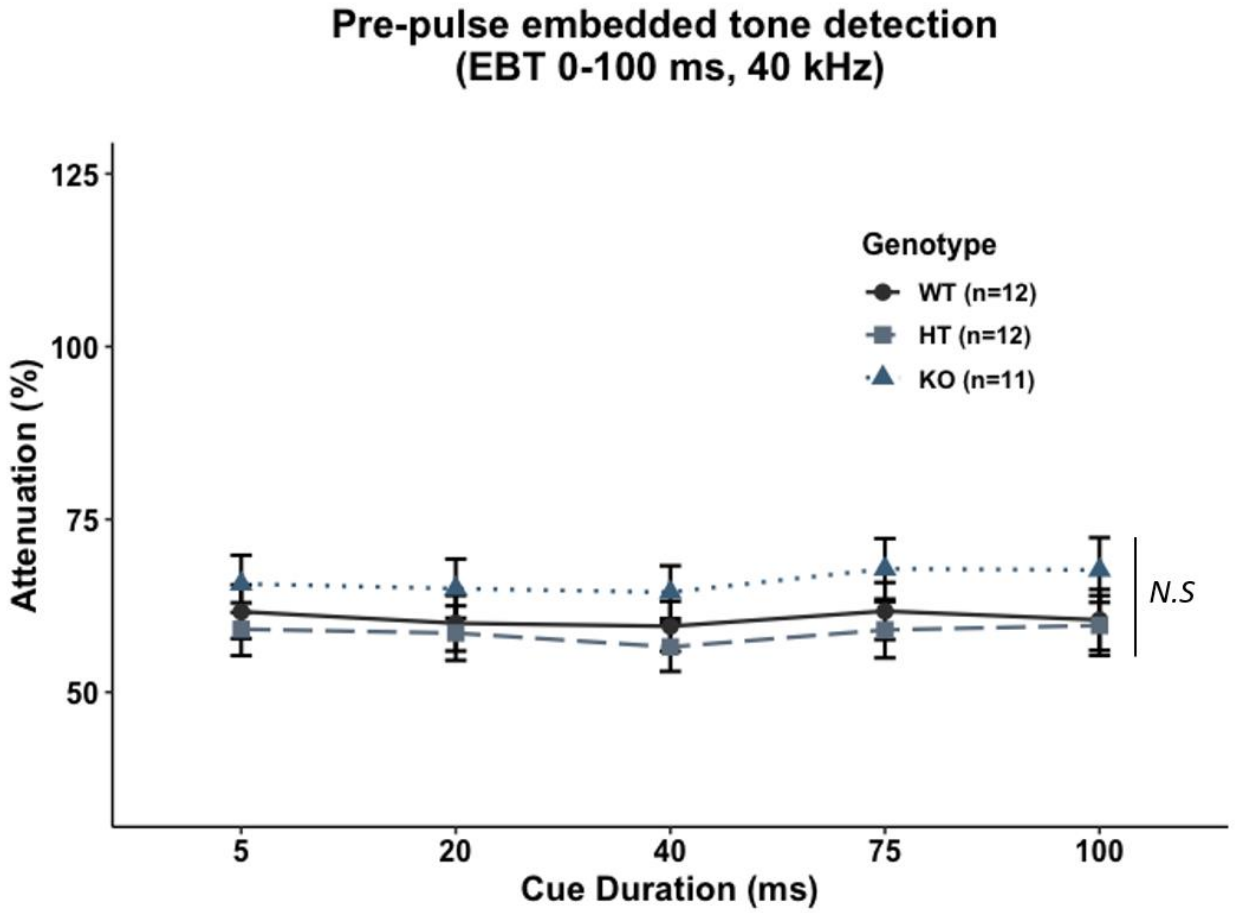

Figure 5 - Embedded Tone (EBT) 0-10 at 40kHz averaged across days
No main effect of Genotype was observed [(Overall): $F(2, 31) = 0.948$, $p > 0.05$]. (*N.S* = non-significant). *Lower attenuation scores indicate better performance*.

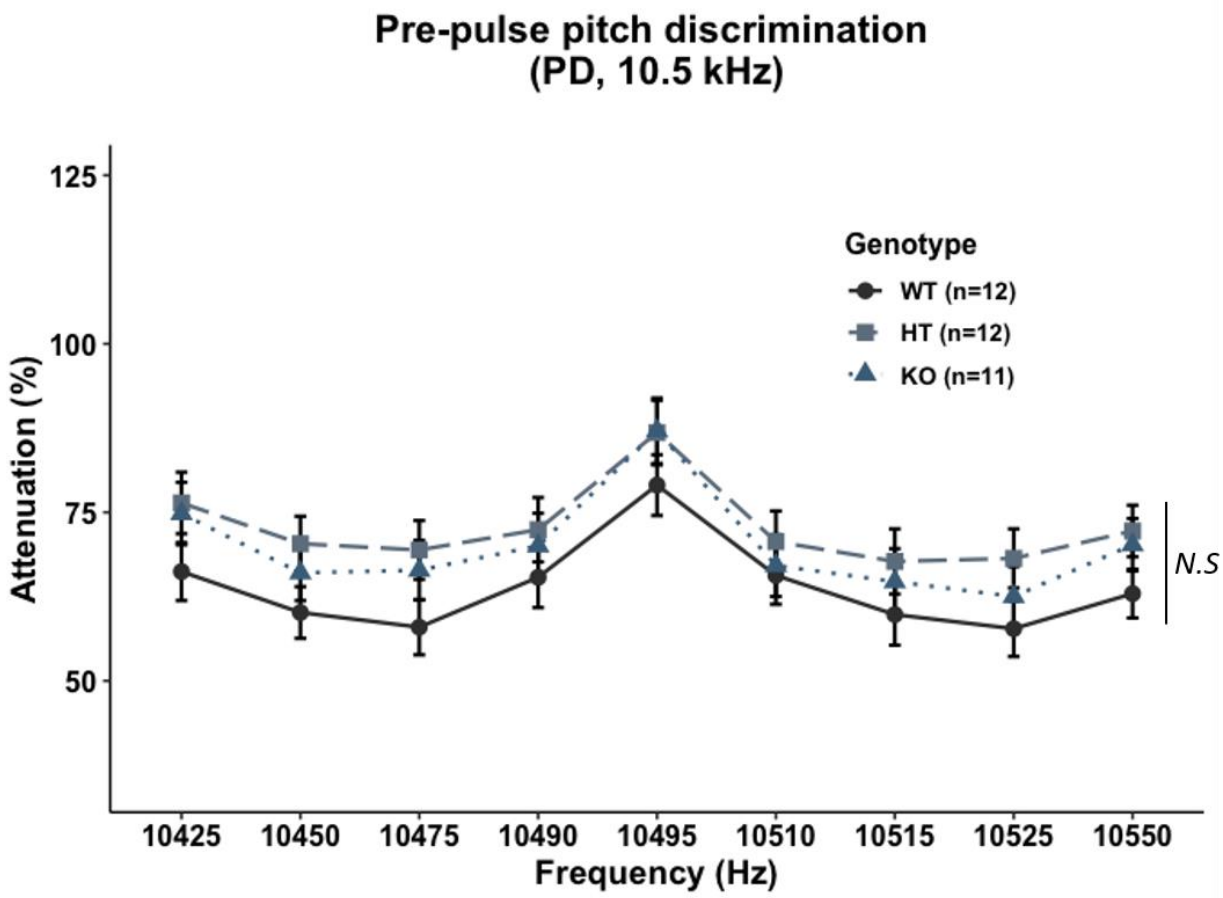

Figure 6 - Pitch Discrimination (PD) at 10.5kHz averaged across days
No main effect of Genotype was observed [(Overall): $F(2, 31) = 1.325$, $p > 0.05$]. (*N.S* = non-significant). *Lower attenuation scores indicate better performance*.

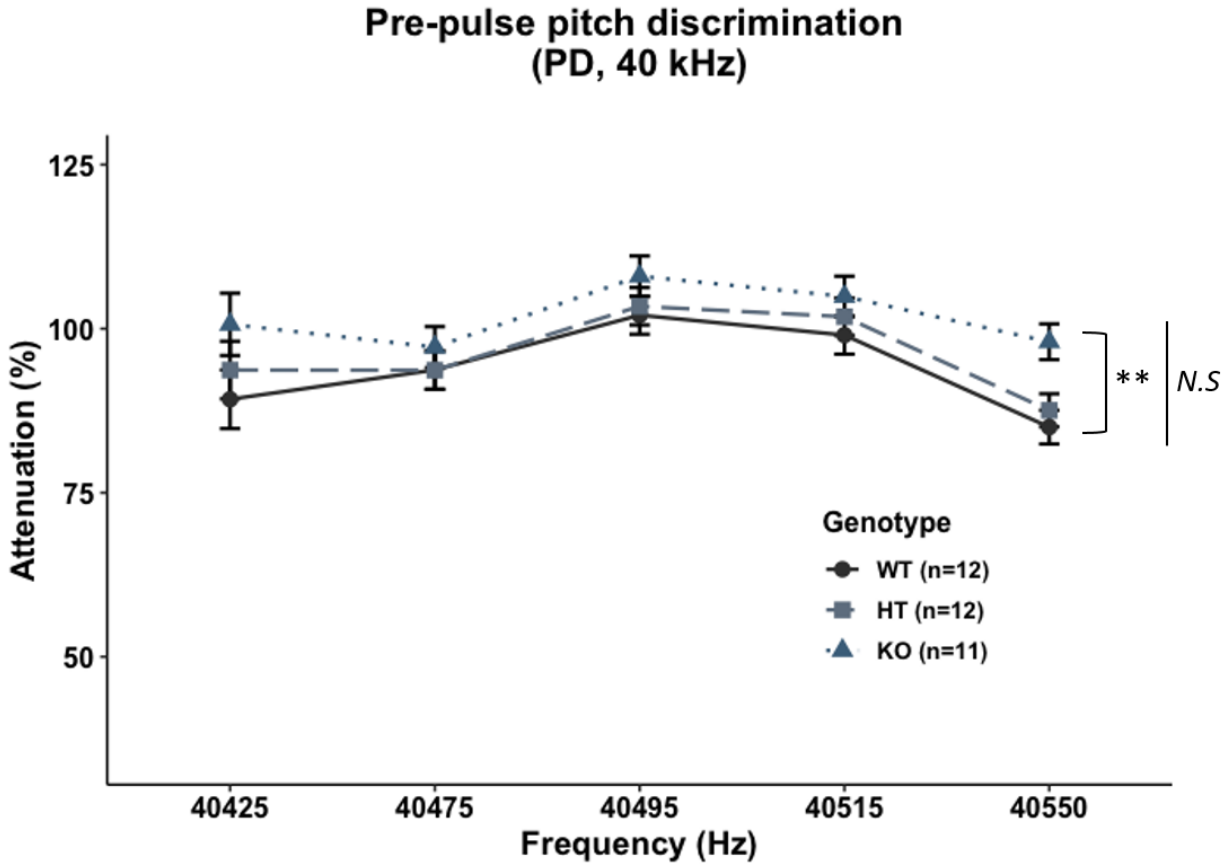

Figure 7 – Pitch Discrimination (PD) at 40kHz averaged across days
No significant effect of Genotype was found [(Overall): $F(2, 31) = 2.335$, $p > 0.05$]. *Ush2a* KO mice, however, displayed poorer performance on PD at 40 kHz compared to WT controls ($p < 0.01$), using NST 40kHz as a covariate. (*N.S* = non-significant, $^{**}p < 0.01$). *Lower attenuation scores indicate better performance*.

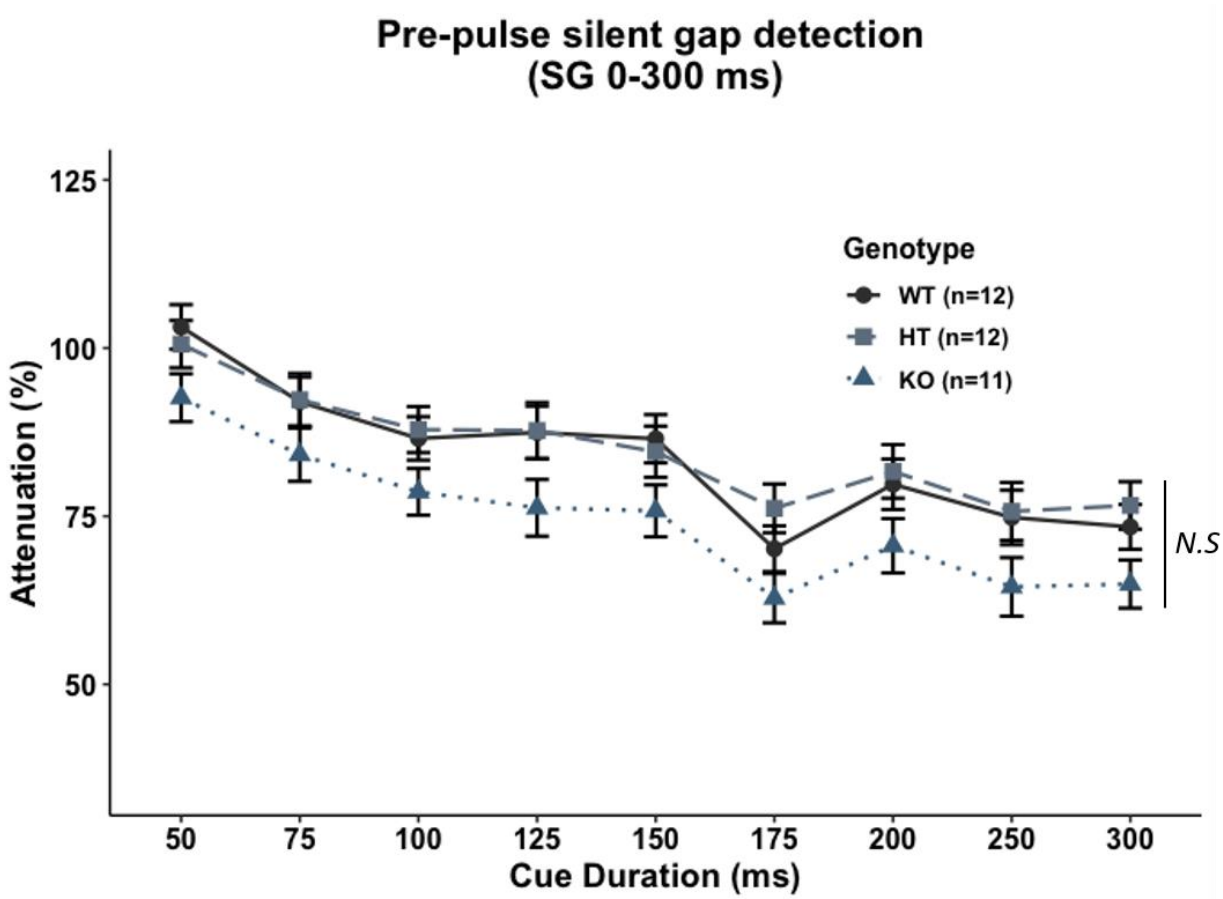

Figure 8 – Silent Gap (SG) 0-300ms averaged across days
No main effect of Genotype was observed [(Overall): $F(2, 31) = 2.497$, $p > 0.05$]. (*N.S* = non-significant). *Lower attenuation scores indicate better performance*.

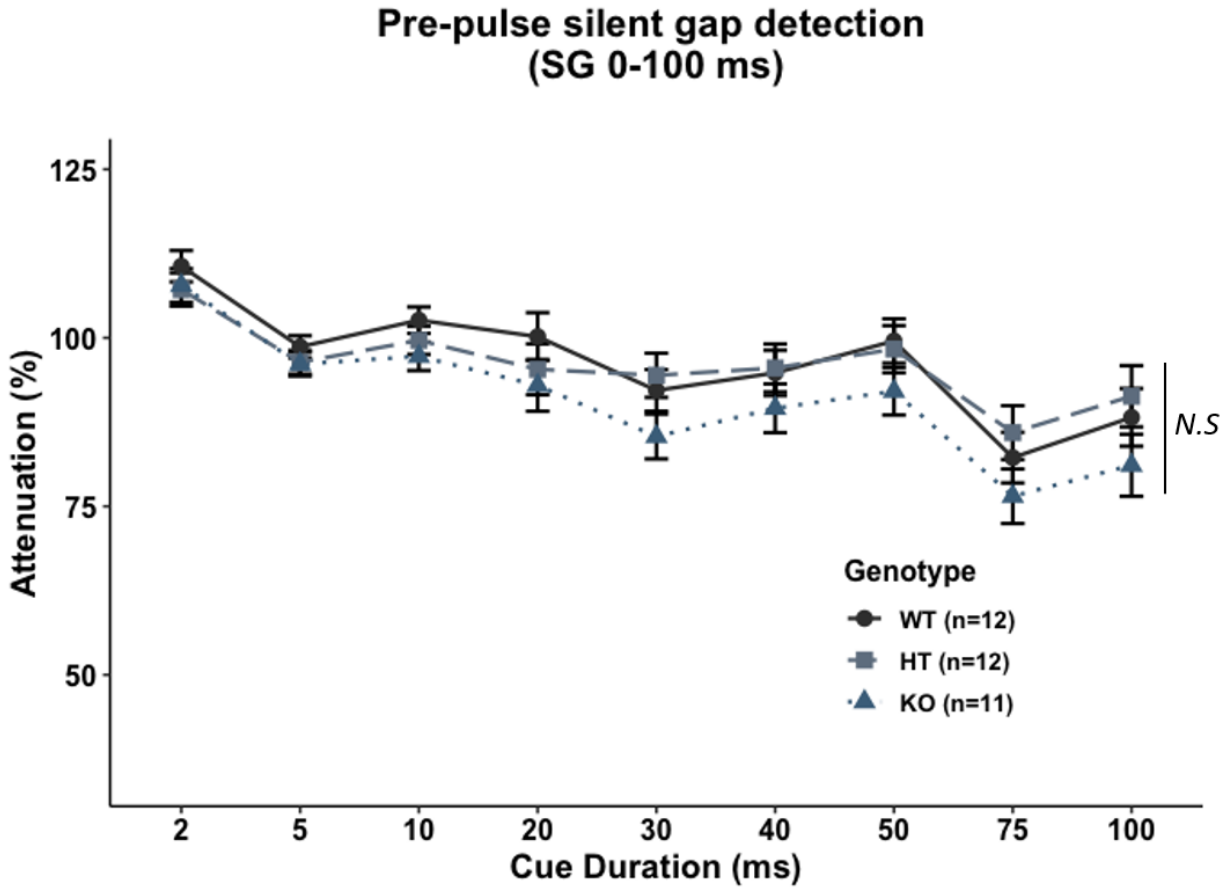

Figure 9 - Silent Gap (SG) 0-100ms averaged across days
No main effect of Genotype was observed [(Overall): $F(2, 31) = 1.378$, $p > 0.05$]. (*N.S* = non-significant). *Lower attenuation scores indicate better performance*.

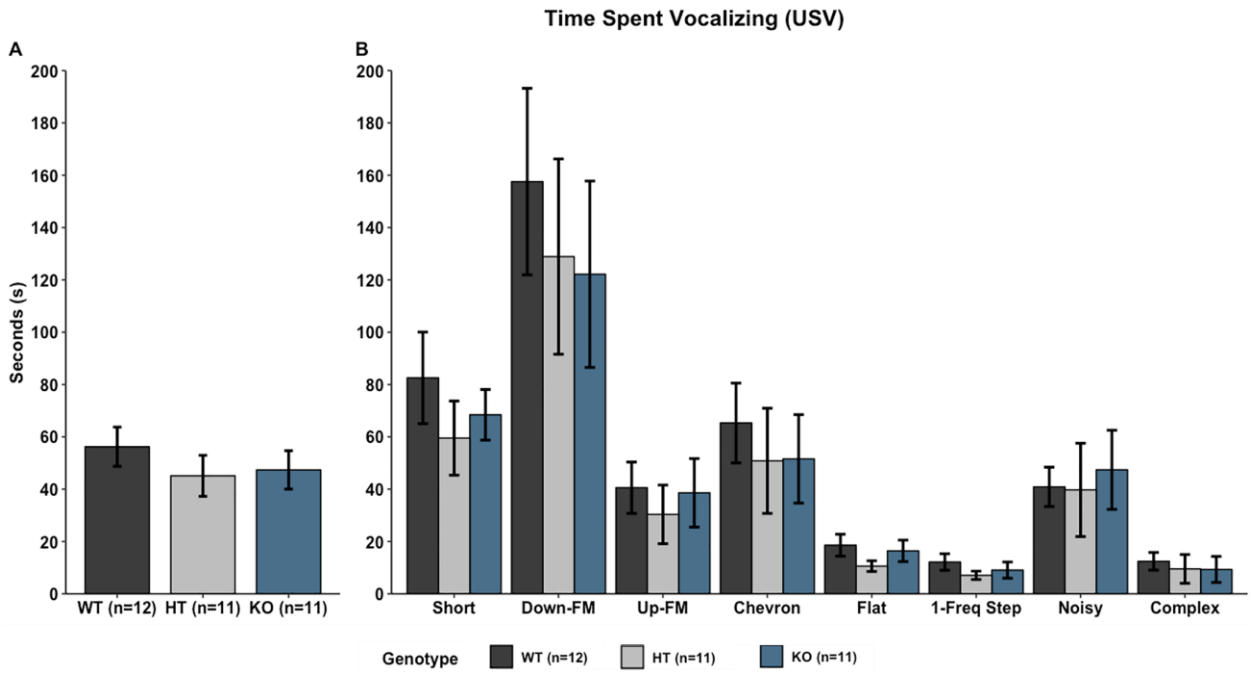

Figure 10 – Ultrasonic Vocalizations (USVs) - Time Spent Vocalizing
(A) No significant effects of Genotype were found on mean time spent vocalizing when collapsed by category. (B) No significant effects of Genotype were found on time spent vocalizing for individual categories.

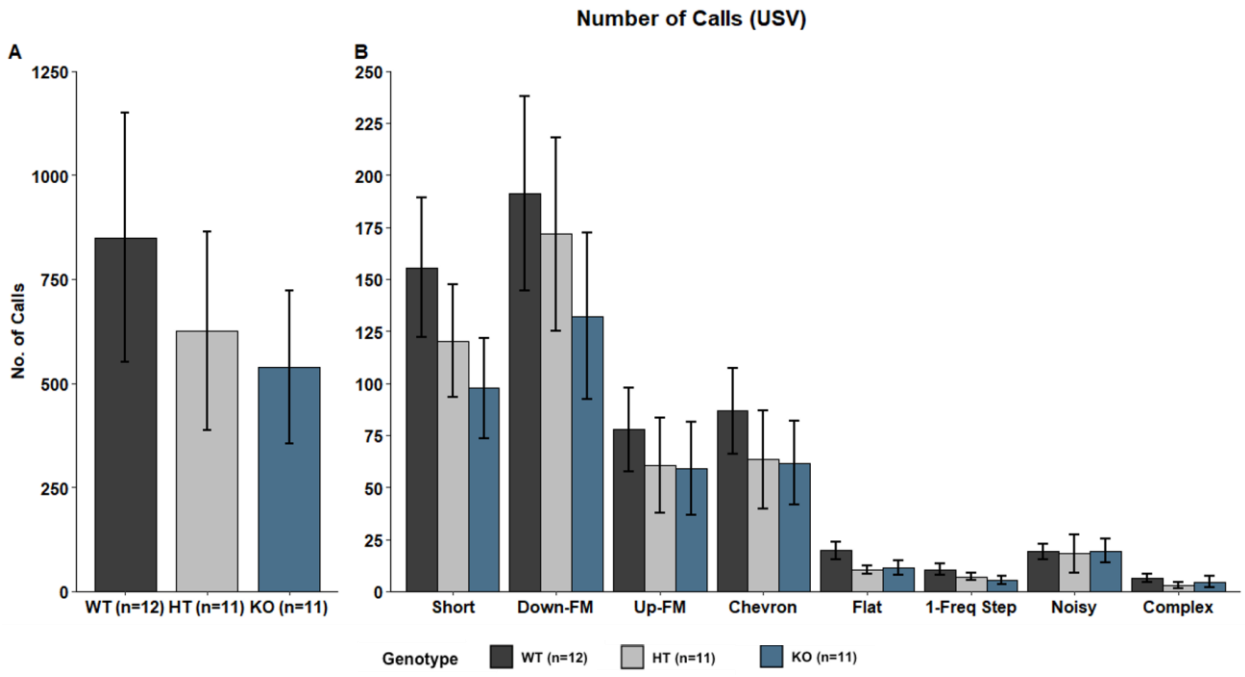

Figure 11 – Ultrasonic Vocalizations (USVs) – Mean Number of Calls
(A) No significant effects of Genotype were found on mean number of calls when collapsed by category. (B) No significant effects of Genotype were found on number of calls for individual categories.

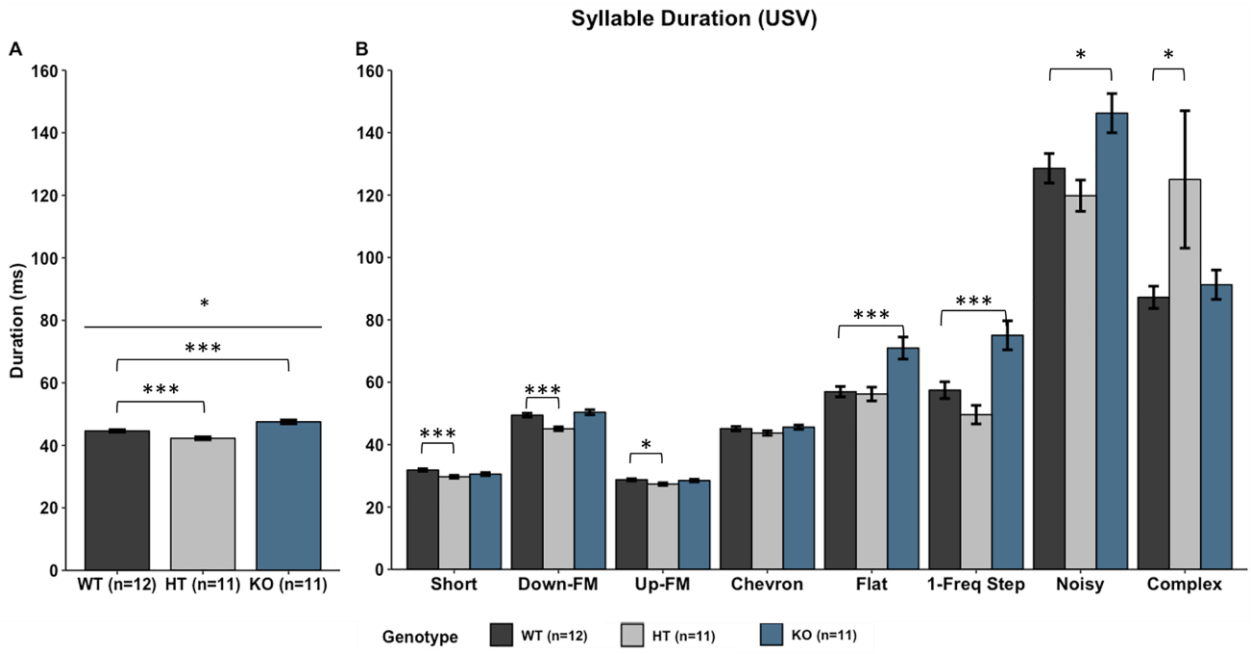

Figure 12 – Ultrasonic Vocalizations (USVs) – Syllable Duration
(A) Overall, *Ush2a* HT mice produced significantly shorter syllables when compared to WT mice [(Overall) $F(2, 16100) = 26.70$, $p < 0.05$]. (B) *Ush2a* HT mice produced significantly shorter Short, Down-FM, Up-FM syllables when compared to WT mice. However, *Ush2a* HT mice produced longer Complex syllables when compared to WT mice. (*$p < 0.05$, ***$p < 0.001$).

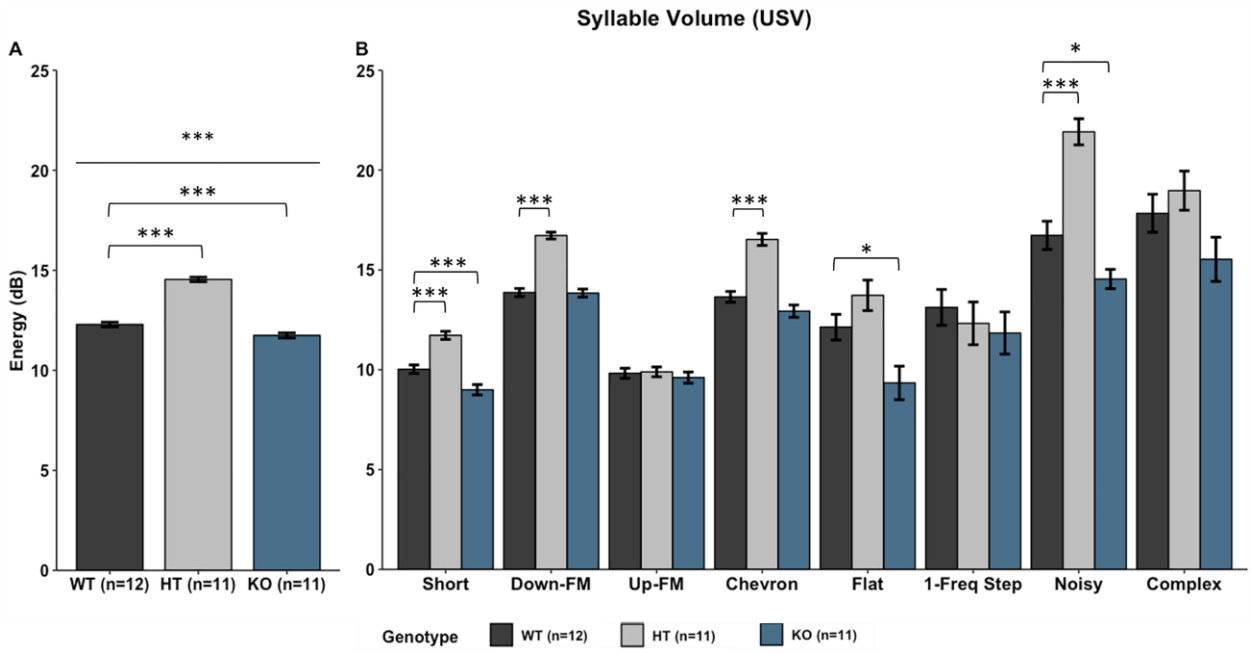

Figure 13 – Ultrasonic Vocalizations (USVs) – Syllable Volume
(A) Overall, *Ush2a* HT mice produced significantly louder syllables when compared to WT mice [(Overall) $F(2, 16100) = 142.54$, $p < 0.05$]. (B) *Ush2a* HT mice produced significantly louder Short, Down-FM, Chevron, and Noisy syllables when compared to WT mice. (*$p < 0.05$, ***$p < 0.001$).

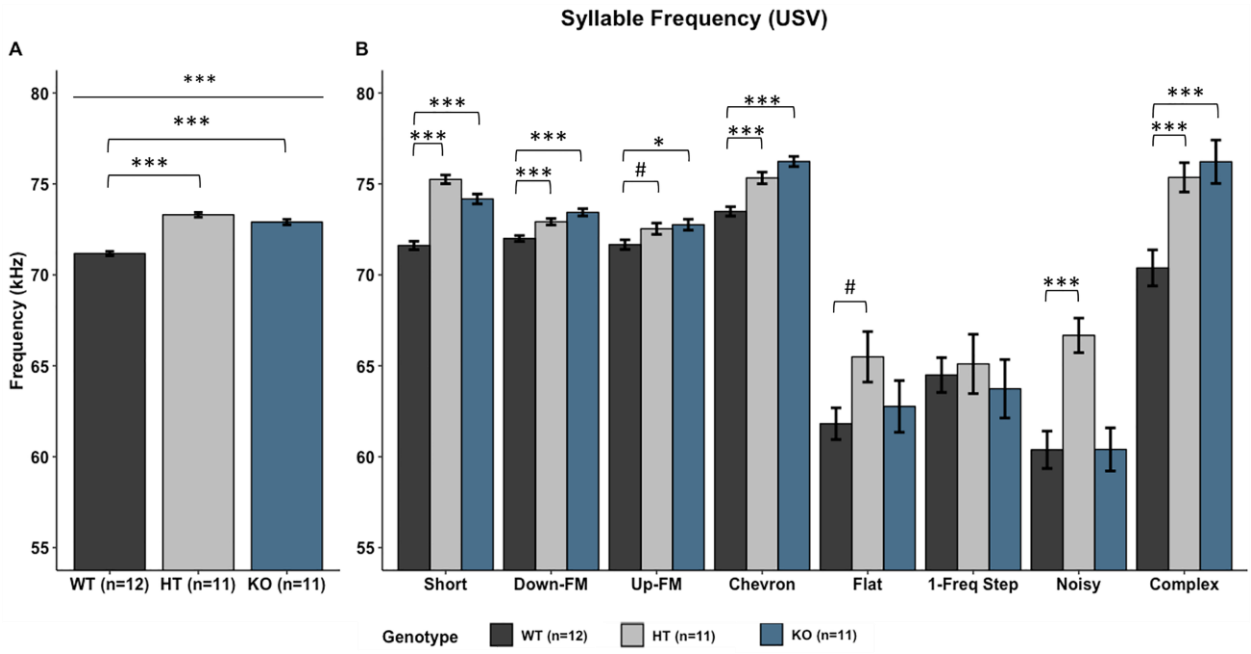

Figure 14 – Ultrasonic Vocalizations (USVs) – Mean Syllable Frequency
(A) Overall, *Ush2a* HT mice produced syllables at significantly higher frequencies when compared to WT mice [(Overall) $F(2, 16100) = 87.476$, $p < 0.05$]. (B) *Ush2a* HT mice produced significantly higher Short, Down-FM, Up-FM, Chevron, Flat, Noisy, and Complex syllables when compared to WT mice. (#$p \leq 0.10$, *$p < 0.05$, ***$p < 0.001$).

Table 1 – Statistics – Syllable Duration per Call Type

Call Type	df	F	p	p (WT vs. HT)	p (WT vs. KO)
Short	2, 4261	6.6133	0.001	0.001	0.107
Down-FM	2, 5633	17.709	0.000	0.000	0.595
Up-FM	2, 2246	3.5819	0.028	0.025	0.872
Chevron	2, 2416	1.6875	0.185	0.317	0.887
Flat	2, 470	10.638	0.000	0.974	0.000
1-Freq Step	2, 259	11.978	0.000	0.173	0.000
Noisy	2, 639	6.1215	0.002	0.482	0.048
Complex	2, 155	4.3314	0.015	0.012	0.933

Table 1 – Statistics summarizing Genotype effect for syllable duration by each call type (df, F, p). p values for WT vs. HT and WT vs. KO comparisons provided. Yellow: significant at $\alpha = 0.05$; Green: significant at $\alpha = 0.10$.

Table 2 – Statistics – Syllable Volume per Call Type

Call Type	df	F	p	p (WT vs. HT)	p (WT vs. KO)
Short	2, 4261	31.692	0.000	0.000	0.004
Down-FM	2, 5633	71.581	0.000	0.000	0.994
Up-FM	2, 2246	0.297	0.743	0.980	0.819
Chevron	2, 2416	37.037	0.000	0.000	0.192
Flat	2, 470	6.9073	0.001	0.296	0.019
1-Freq Step	2, 259	0.4119	0.662	0.828	0.670
Noisy	2, 639	35.123	0.000	0.000	0.032
Complex	2, 155	2.2387	0.110	0.761	0.231

Table 2 – Statistics summarizing Genotype effect for syllable volume by each call type (df, F, p). p values for WT vs. HT and WT vs. KO comparisons provided. Yellow: significant at $\alpha = 0.05$; Green: significant at $\alpha = 0.10$.

Table 3 – Statistics – Mean Syllable Frequency per Call Type

Call Type	df	F	p	p (WT vs. HT)	p (WT vs. KO)
Short	2, 4261	63.479	0.000	0.000	0.000
Down-FM	2, 5633	16.678	0.000	0.000	0.000
Up-FM	2, 2246	4.3098	0.013	0.077	0.019
Chevron	2, 2416	25.817	0.000	0.000	0.000
Flat	2, 470	2.5016	0.083	0.067	0.822
1-Freq Step	2, 259	0.2066	0.813	0.936	0.919
Noisy	2, 639	11.188	0.000	0.000	0.999
Complex	2, 155	9.606	0.000	0.009	0.000

Table 3 – Statistics summarizing Genotype effect for syllable frequency by each call type (df, F, p). p values for WT vs. HT and WT vs. KO comparisons provided. Yellow: significant at $\alpha = 0.05$; Green: significant at $\alpha = 0.10$.

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